PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
P019039WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/ 01161	16/04/1999	17/04/1998
Applicant		
JOHNSON & JOHNSON MEDICAL LIMITED et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. ==

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]/GB 99/01161

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KEIJSERS V. ET AL.,: "Interleukin 10 gene polymorphisms in ulcerative colitis and Crohn's disease" GASTROENTEROLOGY, vol. 114, no. 4. Supp, - 15 April 1998 (1998-04-15) page g3924 XP002113784 the whole document	1-3,20, 21
A	WO 97 39147 A (CEDARS SINAI MEDICAL CENTER) 23 October 1997 (1997-10-23) see whole doc. esp. claims	
A	WO 97 25445 A (CEDARS SINAI MEDICAL CENTER ;UNIV VIRGINIA (US)) 17 July 1997 (1997-07-17) see whole doc. esp. claims and examples	

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 August 1999	10/09/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
T/GB 99/01161

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DI GIOVINE F S ET AL: "Single base polymorphism at -511 in the human interleukin-1beta gene (IL1beta)" HUMAN MOLECULAR GENETICS, vol. 6, no. 1, 1992, page 450 XP002077315 ISSN: 0964-6906 ---	
A,P	COX A ET AL: "AN analysis of linkage disequilibrium in the interleukin-1 gene cluster, using a novel grouping method for multiallelic markers" AMERICAN JOURNAL OF HUMAN GENETICS, no. 62, 17 April 1998 (1998-04-17), pages 1180 1188-1188, XP002077316 ISSN: 0002-9297 see whole doc. esp. table 1 ---	
A	MCDOWELL T L ET AL: "A gentic association between juvenile rheumatoid arthritis and a novel interleukin-1 alpha polymorphism" ARTHRITIS AND RHEUMATISM, vol. 2, no. 38, 1995, page 221 228 XP002077314 ISSN: 0004-3591 the whole document ---	
P,X	WO 98 54359 A (DUFF GORDON ;COX ANGELA (GB); CAMP NICOLA JANE (GB); GIOVINE FRANC) 3 December 1998 (1998-12-03) the whole document -----	1-21

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
T/GB 99/01161

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9739147 A	23-10-1997	AU 2456197 A	07-11-1997
		AU 2725697 A	07-11-1997
		WO 9739146 A	23-10-1997
WO 9725445 A	17-07-1997	AU 1357697 A	01-08-1997
		CA 2242493 A	17-07-1997
		EP 0873425 A	28-10-1998
WO 9854359 A	03-12-1998	AU 7539898 A	30-12-1998

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 02 December 1999 (02.12.99)	
International application No. PCT/GB99/01161	**Applicant's or agent's file reference** P019039WO
International filing date (day/month/year) 16 April 1999 (16.04.99)	**Priority date** (day/month/year) 17 April 1998 (17.04.98)
Applicant HARVEY, Wilson	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

11 November 1999 (11.11.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Marc Salzman

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

REC'D 23 MAY 2000
WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P019039WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/01161	16/04/1999	17/04/1998
International Patent Classification (IPC) or national classification and IPC		
C12Q1/68		
Applicant		
JOHNSON & JOHNSON MEDICAL LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☒ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
12/11/1999	19.05.2000
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Luis Alves, D Telephone No. +49 89 2399 8695

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/01161

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-20 as originally filed

Claims, No.:

1-21 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 1-5 and 7-19 with respect to industrial applicability.

because:

☒ the said international application, or the said claims Nos. as above relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 4, 5, 9-11
	No:	Claims 1-3, 6-8, 12-21
Inventive step (IS)	Yes:	Claims 4, 5, 9-11
	No:	Claims 1-3, 6-8, 12-21
Industrial applicability (IA)	Yes:	Claims 6, 20, 21
	No:	Claims

2. Citations and explanations

see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Reference is made to the following documents cited in the International search report:

D1: WO-A-97/39147
D2: COX A ET AL: 'An analysis of linkage disequilibrium in the interleukin-1 gene cluster, using a novel grouping method for multiallelic markers' AMERICAN JOURNAL OF HUMAN GENETICS, no. 62, 17 April 1998 (1998-04-17), pages 1180 1188-1188.
D3: WO-A-98/54359
D4: KEIJSERS V. ET AL.,: 'Interleukin 10 gene polymorphisms in ulcerative colitis and Crohn's disease' GASTROENTEROLOGY, vol. 114, no. 4. Supp, - 15 April 1998 (1998-04-15) page g3924.

Section I:

Additional observations:

The documents forming the basis of this report include sequence listing pages 1 to 3 as filed on 28 July 1999. These pages shall not form part of the application (Rule 13ter.1(f) PCT).

Section III:

1. Claims 1 to 5 and 7 to 19 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Section V:

1. D1 discloses a method of identifying, diagnosing and screening for inflammatory bowel disease comprising identifying associated TNF polymorphisms (see p.1 and p.8 to p.11). As defined in D1, inflammatory bowel disease encompasses two

chronic diseases: ulcerative colitis and Crohn's disease. Ulcerative colitis is a chronic ulceration in the colon. Consequently, D1 concerns diseases falling within the term "chronic ulcer" used in the present claims.
Thus, the subject-matter of claims 1 to 3, 6 to 8, 12 to 21 is not novel over D1 (Article 33(2) PCT).

2. The subject-matter of claims 4, 5 and 9 to 11 is novel (Article 33(2) PCT) and appears to comply with the requirements of Article 33(3) PCT because an association with the diseases in claims 4 and 5 or between the diseases and polymorphisms in claims 9 to 11 is not made obvious by the cited documents.

3. Document D2, cited in the International search report as an intermediate document, was published on 17 May 1998, which date is also the priority date validly claimed by the present application. Consequently, D2 is not relevant with respect to Article 33(2) and (3) PCT.

4. The subject-matter of claims 6, 20 and 21 appears to be industrially applicable. Claims 1 to 5 and 7 to 19 encompass methods practised on the human body, as can be understood also from claim 6 which, unlike the claims above, is directed to in vitro methods.
For the assessment of the present claims 1 to 5 and 7 to 19 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to methods for the treatment of the human or animal body by surgery or therapy and diagnostic methods practised on the human or animal body.

Section VI:

1. Document D3 is a patent document cited in the International search report as intermediate document. D3 was published on 3 December 1998, filed on 21 May 1998 and claims a priority date of 29 May 1997.
Since the present application validly claims a priority date of 17 April 1998, D3 is not relevant with respect to Article 33(2) and (3) PCT.
However, should the present application be entered into the regional phase, the

above document could be relevant to the question of novelty.

Section VIII:

1. As acknowledged in the description of the present application (see p.6, last paragraph), the basis of the claimed invention is the finding that certain alleles are over-represented in patients with chronic ulcers. Therefore, the said alleles are essential features of the methods. Since independent claims 1 to 3 do not contain this feature they do not meet the requirement following from Article 6 PCT taken in combination with Rule 6.3(b) PCT that any independent claim must contain all the technical features essential to the definition of the invention. The same objection applies to claims 4 and 5.

2. In view of the state of the art it appears obvious to look for associations between polymorphisms in cytokines and chronic ulcers. However, as illustrated for example by D4, such polymorphisms are not necessarily associated with the said diseases. Thus, the mere indication of the polymorphisms in the description of the present application does not provide a basis for the methods claimed because no association with the diseases is shown. Therefore, the subject-matter of claims 1 to 19 is not supported by the description as required by Article 6 PCT, as the description does not show any association between the polymorphisms and the diseases.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68

A1

(11) International Publication Number: **WO 99/54499**

(43) International Publication Date: 28 October 1999 (28.10.99)

(21) International Application Number: PCT/GB99/01161

(22) International Filing Date: 16 April 1999 (16.04.99)

(30) Priority Data:
9808202.7 17 April 1998 (17.04.98) GB

(71) Applicant *(for all designated States except US)*: JOHNSON & JOHNSON MEDICAL LIMITED [GB/GB]; Erskine House, 68–73 Queen Street, Edinburgh EH2 4NH (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: HARVEY, Wilson [GB/GB]; 53 Westwood, Carlton, Skipton, North Yorkshire BD23 3DW (GB).

(74) Agent: JAMES, Anthony, Christopher, W., P.; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD OF ANALYSIS OF CHRONIC WOUNDS

(57) **Abstract**

The present invention relates to methods of determining susceptibility of a patient to developing chronic ulcers such as dermal ulcers, in particular chronic venous ulcers, arterial ulcers, diabetic ulcers and decubitus ulcers (pressure sores). The methods comprise the determination of the polymorphism type of the patient in genes that encode inflammatory cytokines. These methods may also be used to predict the severity of ulcers and the efficacy of the healing response generated by the body.

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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68

A1

(11) International Publication Number: WO 99/54499

(43) International Publication Date: 28 October 1999 (28.10.99)

(21) International Application Number: PCT/GB99/01161

(22) International Filing Date: 16 April 1999 (16.04.99)

(30) Priority Data:
9808202.7 17 April 1998 (17.04.98) GB

(71) Applicant *(for all designated States except US)*: JOHNSON & JOHNSON MEDICAL LIMITED [GB/GB]; Erskine House, 68–73 Queen Street, Edinburgh EH2 4NH (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: HARVEY, Wilson [GB/GB]; 53 Westwood, Carlton, Skipton, North Yorkshire BD23 3DW (GB).

(74) Agent: JAMES, Anthony, Christopher, W., P.; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD OF ANALYSIS OF CHRONIC WOUNDS

(57) Abstract

The present invention relates to methods of determining susceptibility of a patient to developing chronic ulcers such as dermal ulcers, in particular chronic venous ulcers, arterial ulcers, diabetic ulcers and decubitus ulcers (pressure sores). The methods comprise the determination of the polymorphism type of the patient in genes that encode inflammatory cytokines. These methods may also be used to predict the severity of ulcers and the efficacy of the healing response generated by the body.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
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MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
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SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

Method of analysis of chronic wounds

The present invention relates to methods of diagnosing susceptibility to chronic ulcers such as dermal ulcers, in particular chronic venous ulcers, arterial ulcers, diabetic ulcers and decubitus ulcers (pressure sores). These methods may also be used to predict the severity of ulcers and the efficacy of the healing response generated by the body.

The pathogenesis of chronic ulcers at present remains unknown, although many of the physiological mechanisms that initiate and cause persistence of ulcers have been studied closely. For example, reduced oxygen extraction, perivascular fibrin cuffing and trapping of cytokines are all observable features of venous ulcers. However, the link between these physiological disturbances and the pathogenesis of the condition remains elusive.

Venous ulceration alone costs the United Kingdom Health Service about £150 to £600 million each year and affects around 150,000 patients in the United Kingdom. Much of this cost is spent on care in the community, with up to 30% of community nursing time spent on treating leg ulcers. Some chronic ulcers respond rapidly to treatment, whilst others do not; indeed, many fail to heal over periods of several years. Furthermore, an ulcer may increase in size rapidly, or may remain static in terms of its size.

The choice of treatment should ideally be related to the prognosis. For example, if the prognosis is very good, then conservative treatment (such as on an out-patient basis) may be indicated. If the prognosis is poor, then a more interventional approach may be appropriate, involving surgery and skin grafting.

At present, there is no objective prognostic test for the severity of chronic ulcers, neither is there any way to estimate the likely time to healing. It is well known that many factors may influence the course of the disease, and at present it is upon these factors that clinicians and nurses subjectively judge the prognosis. These factors include the nature of the causative disease (for example diabetes, venous insufficiency, arterial insufficiency, ischaemia), patient age, nutritional status, ulcer duration, patient

compliance with treatment, the nature of the treatment, and other inexact criteria (Rijswijk, 1993; Skene *et al.*, 1992).

In many chronic inflammatory diseases, the up-regulation and/or dysregulation of cytokine production in inflamed tissue and wound fluid is thought to contribute both directly and/or indirectly to the pathology of the disease. Cytokines are peptide/protein immunomodulators that are produced by activated immune cells including thymus-derived T lymphocytes, B lymphocytes and monocyte/macrophages and may also be stored (e.g. in platelets) and synthesised by non-immune cells. The cytokines include interleukins, colony-stimulating factors for granulocytes and/or macrophages, tumour necrosis factors, and interferons.

Wound fluid (the exudate from wounds) contains a mixture of serum and tissue-derived proteins, including many cytokines. Its composition is thought to reflect the microenvironment of the wound site. This environment may be different within healing and non-healing chronic wounds; it has been postulated that chronic leg ulcers do not heal because there is a deficit of growth promoting cytokines (Schultz *et al.*, 1991). Conversely, a net excess of growth inhibiting cytokines may also be present; wound fluid from leg ulcers has been reported by several groups to inhibit fibroblast and keratinocyte proliferation (Bucalo *et al.*, 1989; Harris *et al*, 1991; Shakespeare *et al.*, 1991).

While transient inflammation is a key integral stimulatory process in the healing of acute wounds, excessive and prolonged inflammation can lead to tissue breakdown and can cause wound chronicity. In cutaneous animal models of inflammation, the response of the dermis to an intradermal injection of endotoxin (LPS) has been described. Neutrophil recruitment in LPS-induced injury was found to be associated with raised levels of TNFα, IL-1 and IL-8 (Silber *et al.*, 1994).

A recent report indicates that retention fluid from blisters of partial skin thickness burns, which contain relatively large amounts of cytokines and growth factors, have a surprisingly high level of IL-8 (Ono *et al.*, 1995). IL-8 is a potent chemoattractant for

neutrophils and there is convincing data which demonstrates that TNFα-induced transendothelial neutrophil migration is IL-8 dependent (Smart *et al.*, 1994).

The microvasculature of venous ulcers is characterised by pericapillary fibrin cuffs and by plugging of the capillaries by white blood cells. It has been shown that in patients with venous leg ulcers who display this pathological feature, the white blood cells express high levels of TNFα and the authors suggest that this may explain the absence of wound repair in these patients (Claudy *et al.*, 1991).

Two groups (Stacey *et al*, 1995; Harris *et al* (1995) have measured the levels of growth factors and cytokines in chronic leg ulcers from human patients and found that the levels of the inflammatory cytokines (IL-1, IL-6 and TNFα) were all significantly lower in wound fluid from the healing phase, when compared with the initial non-healing phase of the venous leg ulcers. Conversely, there was no alteration in the levels of growth factors such as PDGF, FGF and EGF.

Leg ulcers are also strongly associated with diabetic pathology. In diabetics, it seems that the mechanism of ulceration may be due to heightened levels of circulating inflammatory cytokines. In this respect, Foss *et al* (1992) have shown that serum TNFα levels are significantly higher in insulin-dependent type 1 diabetic patients than in non-diabetic controls. It has been postulated that raised levels of modified lipoproteins that are present in diabetics may stimulate macrophages to synthesise and release significantly higher levels of both IL-1 and TNFα than levels that are found in healthy patients. The release of cytokines from activated macrophages into the sub-endothelial spaces may have a significant role in the promoting the interaction of endothelial cells with mononuclear cells, so causing endothelial damage (Lopes-Virella, 1996).

The role of leukocytes in tissue damage in the liposclerotic skin of venous disease has also been investigated. In a significant number of patients, lipodermatosclerosis (LDS) is the prelude to or is associated with a venous leg ulcer. It has been shown that in severe LDS in the absence of a detectable venous leg ulcer, dermal staining from both IL-1 alpha and IL-1 beta is increased, which is thought to be a contributing factor in the observed progression to venous disease (Wilkinson *et al.*, 1993).

Patients with severe burn injuries are another patient group in which significant effort has been made to understand the contribution of pro-inflammatory cytokines to the healing process. Plasma levels of IL-1, TNFα and IL-6 have been monitored in burn patients throughout the healing process. The results indicate that the systemic cytokine response to burn injury is mainly represented by IL-6 (de Bandt *et al.*, 1994; Papini *et al.*, 1997). A similar study by Yamada *et al* (1996) measured blood levels of TNFα, IL-6 and IL-8. The increased levels of each cytokine were found to reflect the severity of the associated burn injury. In addition, the level of IL-1β has been positively correlated with burn size, thereby implicating this cytokine in the pathogenesis of thermal injury.

In addition to their role in orchestrating the host response to injury, cytokines such as TNFα, IL-1 and IL-6 are key regulators of matrix metalloproteinase and neutrophil elastase synthesis. There is a significant amount of data which suggests that raised levels of matrix metalloproteinases and neutrophil elastase are associated with poor wound healing, particularly in chronic skin wounds such as venous, diabetic, pressure ulcers and severe burn injuries. For instance, TNFα and elastase activity were found in the granulation tissue of venous stasis ulcers although these proteins are barely detectable in acute wounds (Claudy *et al.*, 1991; Wilkinson *et al.*, 1993). Grinnell and Zhu (1994; 1996) have also implicated neutrophil elastase in the delayed healing of chronic skin wounds.

It has also been demonstrated (Schultz *et al,* 1993) that the mitogenicity of fibroblast cultures in acute wound fluid is lost if the experiment is repeated using chronic wound fluid. Since the mitogenicity of chronic wound fluid could be restored in the presence of a protease inhibitor, this suggests that excess protease activity was responsible for the reduction in fibroblast activity.

The balance between matrix deposition and tissue turnover is fundamental in wound healing. It is thought that the balance between proteolytic enzymes and their natural inhibitors contributes to this. A recent report (Bullen *et al.*, 1995) has shown that chronic wounds contained significantly higher levels of gelatinases and the levels of

tissue inhibitors of metalloproteinase (TIMPs) were lower than in healing wounds. This data suggests that excess proteolysis in chronic wounds retards successful healing, and results from an imbalance of proteinase and inhibitors, as well as the presence of higher levels of activated metalloproteinases.

However, despite this fairly detailed knowledge that we now possess regarding the composition of the microenvironment of chronic ulcers, there is no practical test available which enables a clinician to judge the severity of such wounds or the probable success with which the ulcer is likely to heal without treatment. This is considered by the present inventors to be partially due to the marked genetic variation that exists at the multiple genetic loci that control the inflammatory and other immune responses that are involved with chronic ulcers.

Furthermore, currently, there is no way by which it can be predicted whether an individual is likely to be susceptible to chronic ulceration. There thus exists a great need for a reliable, objective test that would allow the identification of individuals who are at risk from contracting a chronic ulcer. Such a test would also be invaluable to allow the prognosis of ulcer severity and/or time to healing and would provide a clinician or nurse with an indication of what kind of treatment regime might be applicable in each case.

The inventors have noted an increased frequency of particular alleles in individuals in both population and family studies, in connection with the incidence of severe chronic ulcers that do not heal. It has been found that there is a link between the polymorphism type of various genes that encode inflammatory cytokines in a patient and the risk that the patient may develop a chronic ulcer. Furthermore, this association can be extended to allow diagnosis of the likely severity of a chronic ulcer, if already partially developed, and the prospective efficacy with which the ulcer will heal. Measurement of these polymorphisms can be made from small samples of patient's tissue, such as blood, and compared with a database of such polymorphisms for prognosis of the ulcer.

Summary of the invention

According to the present invention there is provided a method of determining susceptibility of a patient to developing a chronic ulcer, comprising determining the polymorphism type in genes that encode inflammatory cytokines in the patient. According to a second aspect of the invention there is provided a method of predicting the severity of a chronic ulcer in a patient comprising determining the polymorphism type in genes that encode inflammatory cytokines in the patient. According to a still further embodiment of the present invention there is provided a method of predicting the healing response in a chronic ulcer in a patient comprising determining the polymorphism type in genes that encode inflammatory cytokines in the patient.

Preferably, the chronic ulcer is a dermal ulcer, selected from the group consisting of chronic venous ulcers, pressure sores, decubitis ulcers, diabetic ulcers and chronic ulcers of unknown aetiology.

Polymorphisms are variants in the sequence of a gene within a population. Gene polymorphisms are therefore a mechanism by which individuals may exhibit variations within the range of what is considered to be biologically normal. They may be sequence alterations that are found in populations from different ethnic or geographic locations that, while having a different sequence, produce functionally equivalent gene products. A good example of such sequences are those of the major histocompatibility complex (MHC). Polymorphisms also encompass variations that can be classified as alleles and/or mutations that produce gene products which may have an altered function from that of the normal (wild type) gene product. Polymorphisms also encompass variations which can be classified as alleles and/or mutations which either produce no gene product, an inactive gene product or increased levels of gene product.

According to the present invention, it has been found that at various loci that encode genes for inflammatory cytokines, some allelic variants are over-represented in patients who suffer from chronic ulcers. It is these genetic polymorphisms that give altered

levels or activities of inflammatory cytokines that thus lead to an increased incidence of chronic ulcers, heightened severity and a decreased healing response in afflicted individuals. Such altered levels or activities may directly alter the microenvironment of an ulcer, or may exert downstream effects on molecules that themselves deteriorate the condition of the wound or impair its repair.

This discovery allows the early detection of a predisposition to developing a chronic ulcer and represents a much improved opportunity for medical intervention than treatment of the disease once the symptoms have already commenced. The supervision of a patient over a period of time in which he or she is thought to be at risk from developing a chronic ulcer then allows early diagnosis that may improve prognosis and allow preventative intervention before the clinical symptoms of the disease are noticed. This also means that patients who cannot be differentiated on the basis of their clinical symptoms may be separable on the basis of their genetic disposition to the disease; such analysis allows the development and application of more individual treatments that suit patients with subtle or undetectable differences in their disease state.

In most cases, the genetic polymorphisms that are associated with chronic ulcers cause an increase in the activity or levels of inflammatory cytokines. As discussed above, many research groups have previously attempted to find correlations between levels of inflammatory cytokines in various inflammatory conditions, but no real consensus has emerged as to which cytokines are causative and which are simply the result of increased levels or activities of other cytokines. It is therefore hypothesised that this failure is due to the fact that it is subtle alterations in the activities of inflammatory cytokines that are responsible for changes in patterns of susceptibility to and prognosis of chronic ulcers.

The polymorphisms that are the subject of the present invention are present in any inflammatory cytokine whose activity is altered in the microenvironment of chronic ulcers. Preferably, the polymorphisms are present in the inflammatory cytokines IL-1, IL-6, IL-8 and TNFα, although other suitable candidates will be apparent to those of skill in the art.

Of particular suitability for use in accordance with the present invention are the polymorphisms listed below, which are indicative of increased risk/severity of developing a chronic ulcer.

There are three known IL-1 genes, that form a cluster on human chromosome 2q13. IL-1A and IL-1B produce IL-1α and IL-1β, respectively. IL-1RA binds to IL-1 receptors and acts as a receptor antagonist. The presence of allele 2 of the IL-1A -889 polymorphism or allele 2 of the +3953 polymorphism of the IL-1B gene is a positive indicator of susceptibility to chronic ulcers. This is thought to be due to an elevation of active levels of IL-1 produced by monocytes in individuals that possess these polymorphisms. Individuals that are heterozygous for either of these polymorphisms are at greater risk than those individuals that possess wild type IL-1A or IL-1B loci. Homozygous individuals are at even greater risk. Those individuals who possess both polymorphisms, and are either heterozygous or homozygous for either or both of these polymorphisms are at greatest risk.

A further IL-1B polymorphism herein linked to chronic ulcers is the IL-1B –511 polymorphism. Details of other polymorphic sites in IL-1 genes may be found in the following references: Laurent *et al.*, 1997; Heresbach et al, 1997; Tarnow *et al.*, 1997a; Tarnow *et al.*, 1997b; Cork *et al.*, 1996; Guasch *et al.*, 1996; Clay *et al.*, 1996; Lakemore *et al.*, 1996; Satsangi *et al.*, 1996; Bioque *et al.*, 1995; Crusius *et al.*, 1995; Danis *et al.*, 1995b; van den Veldan *et al.*, 1993; Bailly *et al.*, 1993; Feltes *et al.*, 1993; Jacob *et al.*, 1993; di Giovine *et al.*, 1993; Todd *et al.*, 1993 and Richter *et al.*, 1989.

In transgenic mice that over-produce TNFα, abnormal TNFα production has been shown to contribute to disease initiation and progression of rheumatoid arthritis, systemic inflammatory response syndrome and diabetes (Probert *et al.*, 1996 *J Leukocyte Biol* **59(4)**: 518-525). TNFα is another inflammatory cytokine for which polymorphisms that generate altered activity from normal are herein linked with chronic ulcers, particularly chronic ulcers. An example of such a polymorphism is that at position –308 in the TNFα gene. Further examples of TNFα polymorphisms which the skilled man will be able to apply to the diagnosis of chronic ulcers may be found in the following references: Abraham *et al.*, 1993; Wilson *et al.*, 1992; Pociot *et al.*, 1991;

Seitzer *et al.*, 1997; Brinkman *et al.*, 1997; Demeter *et al.*, 1997; Louis *et al.*, 1996; Bouma *et al.*, 1996; Chen *et al.*, 1996; Fong *et al.*, 1996; Wilson *et al.*, 1995; Danis *et al.*, 1995a; Verjans *et al.*, 1994 and Stokkers *et al.*, 1995.

The gene that encodes IL-6 also contains polymorphisms whose presence can be positively correlated with susceptibility to chronic ulcers. One example is the *Bgl*II mutation (Blankenstein *et al.*, 1989; Fugger *et al.*, 1989a). Further examples may be found in the following references: Murray *et al.*, 1997; Danis *et al.*, 1995a; Stokkers *et al.*, 1995; Toungouz *et al.*, 1994; Shalhevet *et al.*, 1993; Jacob *et al.*, 1993; Titenko *et al.*, 1991; Fugger *et al.*, 1989b and Dawson *et al.*, 1993.

With reference to IL-8, the *Hind*III polymorphism is of use in the diagnosis of susceptibility to chronic ulcers (Fey *et al.*, 1993).

Polymorphisms may also be present in genes that encode receptors for inflammatory cytokines, whose activity is necessary for the effective biological function of the cytokine. Examples of such polymorphisms are the promoter polymorphism of the plasminogen activator inhibitor (PAI-1) gene that causes an altered response to IL-1 (Dawson *et al.*, 1993) and the polymorphisms that are responsible for alternative forms of the human granulocyte colony stimulating factor (G-CSF) that cause changes in growth signal transduction (Ziegler *et al.*, 1991).

Typing of the genetic polymorphisms of a patient are carried out *ex vivo*. Assessment of polymorphism type may be either through the use of specific antibodies directed against the antigenic determinants of the inflammatory cytokines or may be by analysis of the genotype of the patient. Preferably, typing is by genetic analysis of the inflammatory cytokine locus.

In order to ascertain the genotype of a patient, a sample of the DNA of that patient must be available. This sample may be obtained from any tissue of the body. Commonly-used tissues for biopsy are the blood, buccal epithelium, skin or hair. Preferably, the DNA sample is obtained from blood samples. In a preferred embodiment, the DNA is obtained from blood cells obtained from a finger prick of a patient. The blood may be

collected on absorbent paper, or preferably on an AmpliCard™ (University of Sheffield, Department of Medicine and Pharmacology, Royal Hallamshire Hospital, Sheffield, England S10 2JF), also described in Tarlow JW. *et al.* 1994 *Journal of Investigative Dermatology*: **103**: pp387-389.

This embodiment has the advantage of requiring only a small amount of blood and avoids the necessity for venipuncture or a tissue biopsy. However, other means for collecting DNA and determining polymorphism patterns as known in the art can be used.

Molecular DNA typing of the inflammatory cytokine gene locus may be carried out by detection and assignation of the DNA polymorphisms in the inflammatory cytokine gene through the use of various techniques that will be well known to those of skill in the art. There are three preferred methods. These are first the detection of restriction fragment length polymorphisms (RFLPs); second, Southern blotting of PCR-amplified DNA using specific probes; and third, direct sequencing of PCR products. The latter method, which although more laborious is more stringent, is generally the preferred method of the present invention.

RFLPs are changes in a specific DNA (termed a polymorphism if the differences between human individuals occur more frequently than every 10^7 bases) that may be traced using restriction enzymes. When a polymorphism occurs in a consensus sequence that is recognised by a particular restriction enzyme so that this sequence is no longer recognised, the DNA fragments produced by restriction enzyme digestion will be of different sizes. The various possible fragment sizes from a given region therefore depend on the precise sequence of the DNA in the region. This variation in the fragment sizes is termed a restriction fragment length polymorphism (RFLP), and can be visualised by separating the DNA according to its size on an agarose gel.

The individual fragments may be visualised by annealing to a labelled oligonucleotide probe that is specific for the sequence of the fragment of interest. Various methods of labelling the probe will be known by those of skill in the art and will most commonly involve the use of radioactivity or fluorescent or enzymatic tags.

According to the present invention. the more preferred method of detection of polymorphisms is through the amplification of a DNA fragment that is then analysed using probes that are specific for the particular polymorphism of interest. Alternatively the amplified DNA fragment may be sequenced directly. Preferably, the DNA fragment is amplified using the polymerase chain reaction (PCR). The amplified DNA fragment will of course comprise the portion of the inflammatory cytokine gene that contains the polymorphism of interest.

A diagnostic length of DNA may be amplified by PCR using primers raised to conserved DNA sequence in the inflammatory cytokine gene. By a diagnostic length is meant a fragment of sufficient length to allow discernment of the characterising polymorphisms of each inflammatory cytokine antigen type. Thus, the fragment must be of sufficient length to allow an oligonucleotide primer to hybridise specifically with this sequence. As will be apparent to those of skill in the art, this fragment of DNA is of at least 50 bases, preferably 100 bases, and most preferably more than 400 bases in length.

The primers used to amplify the DNA fragment may be designed by anyone of skill in the art so as to be complementary in sequence to the gene sequence that flanks the polymorphism. Preferably the reaction conditions for PCR are as described herein or in Kimura and Sasazuki, 1992.

The PCR product can be purified and immobilised for hybridisation by methods commonly used in the art. The fragment may be purified by submarine gel electrophoresis and immobilised on membranes (Boehringer) as described in Kimura and Sasazuki, 1992.

For analysis by Southern blotting, the purified and immobilised PCR product is challenged with labelled sequence-specific probes. Each specific probe comprises an oligonucleotide of complementary sequence to the particular defining polymorphic region of the inflammatory cytokine locus. These probes are specific for each inflammatory cytokine polymorphism type. Under conditions of a certain stringency, each oligonucleotide will only hybridise to the polymorphic DNA sequence against which it is raised and thus will provide polymorphism typing with much more accuracy than is possible using serological

methods. The conditions of stringency to use will be facile for the man of skill in the art to ascertain (see, for example Sambrook *et* al., 1989; Molecular Cloning: a laboratory manual; Ausubel *et* al., eds., John Wiley & Sons, 1992). A further probe capable of specific binding to all wild type loci may be used as a control.

The method of detection of bound probes may be by any one of the methods commonly used in the art. Preferably the probes themselves are labelled, either by radiolabelling, or by chemical modification, for example using digoxigenin (Kimura and Sasazuki, 1992; Boehringer Mannheim catalogue). Detection may be by autoradiography, or by chemiluminescence, respectively, depending on the system chosen. Most preferably, the invention uses digoxigenin-labelled oligonucleotides.

When using digoxigenin-labelled oligonucleotides, a labelled anti-digoxigenin antibody-enzyme conjugate is used for the detection of oligonucleotide. This specific reaction can be visualised by chemiluminescent detection using an AMPPD substrate in accordance with the manufacturer's instructions (Boehringer Mannheim). In the preferred embodiment of the invention, the conjugated enzyme comprises an alkaline phosphatase conjugate.

A preferred method of detection is by direct sequencing of the PCR products. This method is commonplace and will be well-known to those of skill in the art. Briefly, the initial PCR product is subjected to a second amplification employing an Applied Biosystems sequencing kit, as described in Morrison *et al.* 1993. The product is purified twice using phenol/chloroform and then precipitated using ethanol. For the sequencing reaction, the DNA is loaded onto a 6% polyacrylamide gel, before direct sequencing is performed in both forward and reverse directions (in triplicate) using fluorescence-labelled dideoxynucleotide termination on an Applied Biosystem 373A Automated DNA Sequencer. Alternative sequencing kits, PCR purification kits and automated sequencers are readily commercially available and may be employed in the present invention.

According to a further aspect of the present invention, there is provided a diagnostic kit for typing of the polymorphism type of an inflammatory cytokine locus in a patient. All three detection methods described above lend themselves readily to the formulation of kits that can be used in diagnosis. Such kits will contain reagents suitable for applying the method

of the invention to detect the appropriate polymorphisms and will thus provide the necessary materials to carry out the molecular biological reactions that are described above. These are packaged into suitable containers or supports useful for performing the assay.

The essential components of the assay vary depending upon which embodiment of the invention is to be utilised. Regarding the detection of RFLPs, the essential components of the assay include the restriction enzyme associated with the polymorphism and the specific probe. Additionally, packages containing concentrated forms of reagents and buffers used for hybridisation, prehybridisation, DNA extraction and the like may be included. In particular however, labelled probe, or reagents suitable to form conveniently labelled probe are useful in facilitating the conduct of this method of the invention.

In connection with the amplification of DNA fragments using PCR and their subsequent analysis using specific probes, the essential components of the assay kit will include the thermostable DNA polymerase enzyme associated with amplification of the DNA fragment and a suitable probe. For direct sequencing of PCR products, the essential components are the specific primers, a suitable thermostable DNA polymerase enzyme, ATP, the mixed nucleotide units for extension of the nucleotide chain, and fluorescent-labelled dideoxynucleotide termination products.

All documents mentioned in the text are incorporated herein by reference.

Various aspects and embodiments of the present invention will now be described by way of example and illustrated with reference to the figures. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

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EXAMPLES

Analysis of genetic polymorphisms

The subject's finger was cleaned with antiseptic wipes and the skin was punctured with a sterile lancet. Finger-stick blood samples were collected on DNAase-free blotting paper (Tarlow *et al.* 1994) and analysed blind for polymorphism in the IL-1A gene at position -889 (McDowell *et al.* 1995), in the IL-1B gene at positions –511 (Di Giovine *et al.*1992) and +3953 (Di Giovine *et al.* 1996), the IL-1RA gene intron-Z (Tarlow *et al.* 1993), and the TNFA gene at position –308 (Wilson *et al.* 1992).

A reaction mix excluding Taq polymerase was prepared and 1 mm^2 dried blood spots were added prior to heating at 95°C for 15 min. Taq polymerase (1.25 u. GibcoBRL-UK) was then added and PCR started. All reactions were carried out in 20 mM TrisHCl, 50 mM KCl. 0.2 mM each dNTP and 0.05% W-1 detergent. The $MgCl_2$ and printer concentrations varied in each type of reaction and are detailed below.

Analysis for TNFα

The single G/A base variation polymorphism at –308 in the TNFα gene sequence was identified by PCR amplification of genomic templates. A single base mismatch was incorporated into one of the primers in order to complete a *Nco*I restriction site.

Primer 1: AGG CAA TAG GTT TTG AGG GGC AT

Primer 2: TCC TCC CTG CTC CGA TTC CG

PCR conditions were as follows:

Final concentration of primers: 2μM.

1.5mM MgCl2 was used throughout the reactions.

1 cycle [94° (3 minutes); 60°C (1 minute); 72°C (1 minute)];

35 cycles [94° (1 minute); 60°C (1 minute); 72°C (1 minute)];

1 cycle [94° (1 minute); 60°C (1 minute); 72°C (1 minute)].

Restriction enzyme digestion used 6 units per 30µl reaction mixture of NcoI at 37°C for 8 hours. Sizing was using 2% agarose gels or 8% SDS-PAGE (Laemmli, 1970).

Allele 1 yields 2 fragments of 87bp and 20bp.
Allele 2 contains no *Nco*I site and is thus not digested. Consequently, this allele only yields one 107bp product.

Analysis for IL-1

IL-1A –889

Primer 1: AAG CTT GTT CTA CCA CCT GAA CTA GGC
Primer 2: TTA CAT ATG AGC CTT CCA TG

Final concentration of primers: 0.8 µM;
1mM $MgCL_2$ used through out the reactions.
1 cycle [96'C (2 min);
45 cycles [94°C for 1 min; 50°C for 1min; 72°C for 1 min; 50°C for 1 min.

PCR product is digested overnight at 37°C with 6 units per 30µl reaction of *Nco*I. and restriction pattern visualised by electrophoresis through a 6% PAGE (1.50V for 2.5 hours). This gave products of 83bp+16bp (allele 1) and 99bp (allele 2).

IL-1β –511;

Primer 1: TGG CAT TGA TCT GGT TCA T
Primer 2: GTT TAG GAA TCT TCC CAC TT

Final concentration of primers was 1 µM.
25mM $MgCl_2$ was used throughout the reaction.

1 cycle [95°C for 2 min; 53°C for 1 min; 74°C for 1 min]

35 cycles [95°C for 1 min; 53°C for 1 min; 74°C for 1 min]

Digestion of products was with 3 units *Avo*I per 30µl reaction at 37°C overnight. yields products of 190bp+ 114bp (allele 1) or 304bp (allele 2).

IL-1B + 3953:

Primer 1: CTC AGG TGT CCT CGA AGA ATC AAA

Primer 2: CCT TTT TTG CTG TGA GTC CCG

Final concentrations of primers: 2 µM.

2.5 mM $MgCl_2$ was used throughout the reactions.

35 cycles [95°C for 2 min; 67.5°C for 1 min; 74°C for 1 min]

3 cycles [95°C for 1 min; 67.5°C for 1 min; 74°C for 5 min]

The PCR products were digested with 10 units per 30µl reaction of *Taq*I at 65°C overnight. The resulting products of 12bp + 83bp + 97bp (allele 1) and 12bp+ 182bp (allele 2) are diagnostic.

IL-IRN (intron 2)

VNTR;

Primer 1: CTC AGC AAC ACT CCT AT

Primer 2: TCC TGG TCT GCA GGT AA

Primer concentrations were 1µM.

1.75 mM $MgCl_2$ was used throughout the reactions.

1 cycle [95°C for 1 min]

35 cycles [94°C for 1 min; 60°C for 1 min; 70°C for 2 min]

1 cycle [70°C for 5 mins; 55°C for 5 min]

Electrophoresis in agarose was performed at 90V for 45 min. Allele 1 (4 repeats) was 412bp; allele 2 (2 repeats), 240bp; allele 3 (3 repeats), 326bp; allele 4 (5 repeats), 498bp; and allele 5 (6 repeats), 584bp.

All PCT products were stained with ethidium bromide 0.2μg/ml and visualised under ultraviolet light following electrophoresis. All PCR screening methods used in this study have been extensively validated.

Statistical methods

Data analyses should be performed as follows. The presence or absence of a copy of the less frequent allele for each DNA polymorphism or the presence or absence of a composite genotype formed by combining pairs of DNA polymorphism in the IL-1 gene cluster will be compared with the presence or absence of chronic dermal ulceration. This will involve logistic regression analysis and the calculation of odds ratios with the appropriate confidence interval. The strength of association will be assessed by the χ^2 test or Fisher's exact test. A Bonferroni correction (Miller, 1981) will be applied to account for multiple comparisons. All analyses may be performed with the SAS statistical package.

A similar analysis should be performed looking at the severity (defined on a categorical scale) of the ulceration.

CLAIMS

1. A method of determining susceptibility of a patient to developing a chronic ulcer, comprising determining the polymorphism type of the patient in genes that encode inflammatory cytokines.

2. A method of predicting the severity of a chronic ulcer in a patient comprising determining the polymorphism type of the patient in genes that encode inflammatory cytokines.

3. A method of predicting the healing response in a chronic ulcer in a patient comprising determining the polymorphism type of the patient for inflammatory cytokines.

4. A method according to any one of claims 1 to 3, wherein the chronic ulcer is a dermal ulcer.

5. A method according to claim 4, wherein the dermal ulcer is selected from the group consisting of venous ulcers, pressure sores and decubitis ulcers.

6. A method according to any one of claims 1 to 5 wherein the method is carried out *in vitro*.

7. A method according to any one of the previous claims wherein the inflammatory cytokine comprises any one of interleukin 1, interleukin 6, interleukin 8 and tumour necrosis factor alpha.

8. The method according to claim 7, wherein the inflammatory cytokine comprises either of interleukin 1 or tumour necrosis factor alpha.

9. A method according to claim 8, wherein the presence of the +3953IL-1B polymorphism is diagnostic or prognostic for chronic ulcers.

10. A method according to claim 8, wherein the presence of the IL-1A -889 polymorphism is diagnostic or prognostic for chronic ulcers.

11. A method according to claim 8, wherein the presence of the +3953 IL-1B and the IL-1A -889 polymorphisms is diagnostic or prognostic for chronic ulcers.

12. The method of any preceding claim wherein the analysis is carried out by:

(a) digesting genomic DNA from a patient to a diagnostic fragment length;

(b) probing the DNA fragment with a probe specific for a polymorphism type, and

(c) detecting the bound probe.

13. The method of any one of claims 1 to 11, comprising the following steps:

(a) amplifying a diagnostic length DNA fragment of an inflammatory cytokine from DNA samples isolated from patients,

(b) probing the amplified DNA sample with a probe specific for an inflammatory cytokine polymorphism type and

(c) detecting the bound probe.

14. The method of any one of claims 1 to 11, comprising the following steps:

(a) amplifying a diagnostic length DNA fragment of the gene encoding an inflammatory cytokine from DNA samples isolated from patients,

(b) performing a second (nested) amplification to produce greater quantities of specific DNA, and

(c) sequencing the amplified DNA fragment in order to analyse the precise polymorphism type of the gene.

15. The method according to any one of claims 12 to 14 wherein the patient DNA is prepared from a blood sample.

16. The method according to either of claims 12 or 13, wherein the probe is detected using chemiluminescence.

17. The method according to either of claims 12 or 13, wherein the probe is detected by autoradiography.

18. Use of polymorphism typing for inflammatory cytokines in a method of determining susceptibility to, predicting the severity of and/or healing response of chronic ulcers in a patient.

19. Use according to claim 18, wherein said patient is a human patient.

20. A diagnostic kit for use in accordance with any one of the methods of previous claims 1-15 comprising a thermostable DNA polymerase enzyme, specific primers that are complementary to a gene encoding an inflammatory cytokine, ATP, mixed nucleotide units for extension of the nucleotide chain, and fluorescent-labelled dideoxynucleotide termination products.

21. A diagnostic kit for use in accordance with any one of the methods of claims 1-15 comprising a thermostable DNA polymerase enzyme, specific primers that are complementary to a gene encoding an inflammatory cytokine, ATP, mixed nucleotide units for extension of the nucleotide chain, a restriction enzyme

associated with a polymorphism associated with a gene encoding an inflammatory cytokine, a specific probe and concentrated forms of reagents and buffers useful in hybridisation, pre-hybridisation and DNA extraction.

INTERNATIONAL SEARCH REPORT

International Application No
P[redacted]GB 99/01161

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KEIJSERS V. ET AL.,: "Interleukin 10 gene polymorphisms in ulcerative colitis and Crohn's disease" GASTROENTEROLOGY, vol. 114, no. 4. Supp, - 15 April 1998 (1998-04-15) page g3924 XP002113784 the whole document ---	1-3,20, 21
A	WO 97 39147 A (CEDARS SINAI MEDICAL CENTER) 23 October 1997 (1997-10-23) see whole doc. esp. claims ---	
A	WO 97 25445 A (CEDARS SINAI MEDICAL CENTER ;UNIV VIRGINIA (US)) 17 July 1997 (1997-07-17) see whole doc. esp. claims and examples ---	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 August 1999	10/09/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

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INTERNATIONAL SEARCH REPORT

Int[illegible]nal Application No
P[illegible]B 99/01161

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropnate, of the relevant passages	Relevant to claim No.
A	DI GIOVINE F S ET AL: "Single base polymorphism at -511 in the human interleukin-1beta gene (IL1beta)" HUMAN MOLECULAR GENETICS, vol. 6, no. 1, 1992, page 450 XP002077315 ISSN: 0964-6906 ---	
A,P	COX A ET AL: "AN analysis of linkage disequilibrium in the interleukin-1 gene cluster, using a novel grouping method for multiallelic markers" AMERICAN JOURNAL OF HUMAN GENETICS, no. 62, 17 April 1998 (1998-04-17), pages 1180 1188-1188, XP002077316 ISSN: 0002-9297 see whole doc. esp. table 1 ---	
A	MCDOWELL T L ET AL: "A gentic association between juvenile rheumatoid arthritis and a novel interleukin-1 alpha polymorphism" ARTHRITIS AND RHEUMATISM, vol. 2, no. 38, 1995, page 221 228 XP002077314 ISSN: 0004-3591 the whole document ---	
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[illegible]nal Application No
P[illegible]B 99/01161

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		WO 9739146 A	23-10-1997
WO 9725445 A	17-07-1997	AU 1357697 A	01-08-1997
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		EP 0873425 A	28-10-1998
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